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THE EFFECT OF ZINC DEFICIENCY ON THE METABOLISM OF INDOLEACETIC ACID IN THE TOMATO PLANT

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
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THE EFFECT OF ZINC DEFICIENCY ON THE METABOLISM OF INDOLEACETIC ACID IN THE TOMATO PLANT

INTRODUCTION

The reduced content of indoleacetic acid in zinc deficient tomato plants has three possible explanations. These are: firstly, there may be enhanced destruction of indoleacetic acid; secondly, there may be reduced supply of precursors, and thirdly, both of the above possibilities may be operative. Although previous work indicated that the third possibility is the most plausible, emphasis has recently been placed on the effects of zinc deficiency on the synthesis of indoleacetic acid. This emphasis is due to the discovery that in Neurospora, the enzyme responsible for the synthesis of tryptophan is specifically affected by zinc deficiency (40). In higher plants tryptophan is a known precursor of indoleacetic acid, and it is also known that the content of tryptophan is reduced in zinc deficient plants (50, 62).

The work described in this thesis is largely devoted to the search for an enzyme which catalyses the synthesis of tryptophan in higher plants, to characterize the enzyme, and to discover the effects of zinc deficiency on the enzyme. The remainder of the thesis describes work on the effects of zinc deficiency on other reactions important in the metabolism of indoleacetic acid.

Though the problem of zinc deficiency is not solved, this work has extended knowledge of the effects of the deficiency, and permits the suggestion of fruitful avenues for future research.

LITERATURE REVIEW

Historical

As an agricultural problem, zinc deficiency was solved in the early 1930's, when it was found that the beneficial effects of iron sulfate treatment were due to impurities of zinc. Hoagland (30) has summarized the early work on zinc deficiency; his examples show the effect to be widespread geographically, and in the number of plants that are naturally affected. Symptoms of zinc deficiency can be alleviated in several ways: zinc coated nails may be driven into affected trees, zinc salts may be applied to the soil, and solutions of zinc salts may be sprayed on the leaves of the affected plants.

Woodbridge (74), in a recent report of zinc deficiency of fruit trees in the Okanagan Valley of British Columbia, states that zinc sulfate spray was the most effective method of treatment.

Scope of Investigations on Zinc Deficiency

In addition to agricultural aspects, zinc deficiency has received considerable attention as a problem of academic interest. In spite of the large amount of work done, the precise function of zinc in the metabolism of higher plants is still not understood. These investigations may be divided into three sections: Cytological, Physiological, and Biochemical.

Cytological The major contributions to the cytological study of zinc deficiency have been made by Reed (45, 46, 47).

Zinc deficiency in apricot and peach trees is a naturally occurring problem in California. Reed (45) studied the effects of zinc deficiency on the cells of the vegetative buds of these trees. He found that there was a general condition of hypoplasia in the cells, there was

an accumulation of phenolic substances in the vacuoles, an increase in cell size, and an inhibition of cell multiplication. This inhibition of meristematic activity has also been noted by Carlton (10) working with tomato plants. In connection with this point, it is relevant to mention the report of Fujii (14) that zinc is present in the nucleoli of starfish oocytes. He also found that when mitosis proceeds in sea-urchin eggs, the zinc initially present in the nucleoli passes into the chromosomes. It is possible that zinc is similarly associated in higher plants.

A later paper by Reed and Dufrenoy (46) reported that in the cells of zinc deficient apricots the colloids, instead of being dispersed as in normal cells, were aggregated into coacervates. These masses were largely made up of catechol, and had a boundary layer which was phospholipid in nature. A further study by Reed (47) using cytochemical techniques with zinc deficient tomato plant tissues indicated that there was serious interference with phosphorus metabolism. Greater amounts of inorganic phosphate were found in the extra-stelar region and phloem of the affected plants. Other derangements discovered by Reed were that the activity of the enzymes phosphatase and polyphenol oxidase was increased, and that the activity of dehydrogenases was reduced. The content of reducing sugars was greater in the affected plants, and there was less sucrose and starch. This effect on carbohydrate metabolism was the same as had been discovered by Bean (4).

Physiological Zinc deficiency may be found in plants grown in soil which, on analysis, is found to contain ample zinc for the needs of the plant. Hoagland (30) states that the zinc may be bound in the crystal structure of soil colloids, and he also mentions evidence that micro-organisms bind zinc. Soil which previously gave rise to zinc deficiency supplies adequate zinc after sterilization; zinc deficiency is again produced when a small

amount of the unsterilized soil is added to the sterilized soil. A further consideration pointed out by Hoagland is that certain plants, though requiring zinc in comparable amount to other species, are able to derive sufficient zinc from soil that gives rise to a condition of zinc deficiency in the other species. Alfalfa is quoted as an example of a plant which apparently has greater ability to derive zinc from the soil. Arnon has said that in some peach orchards enough zinc has been applied to the soil to theoretically supply the plants for 40,000 years, and yet the plants still show signs of zinc deficiency. It is for this reason that foliar application of zinc salts is frequently the best way of curing zinc deficiency, (Arnon, 2).

Bergh (7) has found that when pea plants are allowed to take up radioactive zinc for a period of 24 hours, the highest concentration of zinc is found in the roots and the lowest in the old blades. When the plant material was fractionated and analysed for the zinc it was found that in the root 77% of the radioactive zinc was insoluble in water, alcohol or ether, whereas in the upper parts of the plant only 20-30% of the radioactive zinc was insoluble in the same solvents. The binding of zinc in plant tissues will be mentioned again later.

Some attention was paid by Bean (4) to the effect of zinc deficiency on nitrogen nutrition. Finding that nitrates accumulated in zinc deficient plants he considered that the reduction of nitrate may have been interfered with. However, when zinc deficient plants are supplied with ammonium salts as a nitrogen source in place of nitrates, deficiency symptoms appeared just as soon and were just as severe. Nevertheless, the effect of zinc deficiency on nitrogen metabolism is pronounced. Bean (4) found that protein nitrogen is reduced, and soluble nitrogen in the form of amino acids was increased, in zinc deficient material. The close

relationship between zinc and nitrogen is emphasized by the work of Ozanne (43). He found that when the supply of zinc was low, zinc deficiency symptoms were much more severe when the nitrogen supply was high. This was true no matter whether nitrogen was supplied as sodium nitrate, ammonium nitrate, ammonium sulfate, or l-asparagine. Zinc concentration was positively correlated with protein concentration in the roots. The proportion of absorbed zinc that was translocated to the plant tops was negatively correlated to the amount of protein in the roots. Ozanne concludes that increased nitrogen supply causes zinc to be retained in the roots as zinc-protein complexes, so accentuating deficiency symptoms in the plant tops.

The work of Smith (51) is relevant to a discussion of zinc deficiency in relation to nutrition. When tomato plants were grown with insufficient supply of zinc several other elements were adversely affected. The content of nitrogen, potassium, manganese, molybdenum, and sulfate were reduced below that of the control plants at statistically significant levels.

Another physiological factor which seems to be of great importance in zinc deficiency is light. Hoagland (30) mentions that in citrus trees it is often seen that zinc deficiency symptoms are more pronounced on the sunny side of the tree. In greenhouse plants deficiency can be relieved by shading the plants with cheesecloth, or by removing them to a place where the light intensity is less. Stout (55) allowed tomato plants to grow in culture solution low in zinc which was radioactive, and when deficiency showed he transferred the plants to the laboratory where the light intensity was less. The recovery of the plants was associated with translocation of the zinc to the upper parts of the plant. The implication is that zinc-protein complexes are broken down under reduced light so releasing zinc which is then translocated.

In the course of a study of the relationship between zinc and auxin, Skoog (50) examined the effect of different wavelengths of light. Tomato plants were grown in solutions low in zinc; some plants were grown under red cellophane, and others were grown under blue cellophane. The plants thus grown in red light showed considerable freedom from the symtoms of zinc deficiency, whereas the plants grown in blue light developed deficiency symtoms equal to those of plants grown in sunlight. It is therefore apparent that the light enhancement of zinc deficiency is principally due to light of the shorter wavelengths. Bean has found that the activity of peroxidase is increased in zinc deficient plants. This increase in activity is less pronounced when the zinc deficient plants are protected from intense light.

When Crane (11) found that the accumulation of niacin in tomato leaf disks was enhanced by light, he considered that the finding might be applicable to the interaction of zinc deficiency and light intensity. The work he did to test this possibility (12) showed that zinc deficiency did not result in a significant reduction of ability to synthesize niacin in the light.

The effect of zinc deficiency on two other physiological processes may be mentioned. Firstly respiration; Tsui (64) compared the respiratory ability of leaf segments of normal and zinc deficient tomato plants. He found no significant difference and concluded that zinc plays no direct part in the respiratory system of higher plants. In view of subsequent discoveries of the effect of zinc deficiency on several oxidative enzymes, Tsui's finding is surprising, and his conclusion is certainly questionable.

Secondly, a paper by Tsui (63) examines water content and osmotic pressure of zinc deficient and healthy tomato plants. Whereas

the water content of deficient plants was reduced, the osmotic pressure of the cell sap was greater than that of the healthy plants. This discovery is in accord with the condition of hypoplasia in the cells of deficient plants as found by Reed (45). It is also in agreement with the knowledge that the content of auxin is reduced in zinc deficient plants (Skoog, 50; Tsui, 62) and the knowledge that one of the primary effects of auxin is on the cell wall.

Biochemical The discussion of biochemical investigations of the effects of zinc deficiency in higher plants may be divided into three sections: zinc containing enzymes, miscellaneous enzymes, and enzymes involved in the metabolism of indoleacetic acid.

Zinc containing enzymes a) In 1940 Keilin and Mann (33) established that the carbonic anhydrase of red blood corpuscles is a zinc containing enzyme, the activity of the enzyme being proportional to the zinc content of the preparation. Carbonic anhydrase was later found to be present in green plants (Day and Franklin, 13; Bradfield, 8), and subsequently the enzyme was characterized (Waygood and Clendenning, 68; Byerrum and Lucas, 9). The speculation that the carbonic anhydrase of higher plants is also a zinc containing enzyme was inevitable. This hypothesis was tested by Wood and Sibly (72), and they found that the activity of carbonic anhydrase was in fact reduced in zinc deficient oat plants. However, they concluded that the effect was not caused by an insufficient zinc supply to activate an apoenzyme, but a blocking of protein synthesis so affecting the protein moiety. This conclusion was endorsed by McElroy and Nason (39). Kondo et al. (35,36) reported the presence of carbonic anhydrase in several vegetables, and also reported the isolation and purification of the enzyme. They found that the purified enzyme did not contain zinc or any other metals detectable by the dithizone test, and by spectrographic analysis. It

therefore appears that carbonic anhydrase may be discarded as a possible line of research to discover the primary effect of zinc deficiency.

b) According to Warburg and Christian as cited by Reed (47), aldolase (zymohexase) of yeast is a metal protein containing 1 gram atom of copper and 1 gram atom of zinc per 3,000,000 grams of protein. The activity of aldolase in zinc deficient oat plants has been measured by Quinlan-Watson (44). He found that zinc deficiency led to decreased activity of aldolase in oat plants and in subterranean clover. Copper deficiency had no effect on the same enzyme. Quinlan-Watson considers three possibilities as explanations for the result: zinc may be a constituent of the apo-enzyme; zinc may be concerned with the synthesis of proteins, including aldolase; zinc may function as a co-enzyme or be in association with one. In connection with the work of Quinlan-Watson, McElroy and Nason (39) state that "far more evidence would be necessary to show this enzyme to be a zinc protein, despite the temptation of analogy with the suggested metallo-nature of aldolase of yeast". In view of the multiple effects of zinc deficiency, the caution of McElroy and Nason is understandable. The only way to avoid the reservations of McElroy and Nason would be to isolate and purify the enzyme in question, and correlate its activity with zinc content in the manner followed by Keilin and Mann (33) in their investigation of carbonic anhydrase.

c) This purification method has been followed by Vallee et al. (66), studying yeast alcohol dehydrogenase. They find that the enzyme is a metallo-enzyme containing four moles of zinc firmly bound to one mole of protein. The enzymic activity is directly dependent on zinc content. The alcohol dehydrogenase of Neurospora is reduced under conditions of zinc deficiency (Nason et al., 41). It is therefore tempting to consider that the alcohol dehydrogenase of higher plants is a zinc containing enzyme;

this hypothesis has not been reported as having been tested in the literature although Reed (47), by virtue of qualitative tests, decided that dehydrogenases in general were less active in zinc deficient tomato plants.

To summarize this section on zinc containing enzymes, it can be said that it is not satisfactory merely to show that a certain enzyme is reduced in its activity to prove that the enzyme is a zinc containing enzyme. It must be isolated, purified, and its activity shown to correlate with zinc content.

Miscellaneous enzymes The effect of zinc deficiency on several other enzymes is shown in the following table.

Table 1. Effects of Zinc Deficiency on Enzyme Activity

Enzyme	Material	Author
Increased Activity		
Peroxidase	Tomato plant	Bean (4)
"	"	Skoog (50)
"	"	Nason et al. (42)
Polyphenol oxidase	"	Reed (47)
Phosphatase	"	"
"	"	Nason et al. (42)
Ascorbic acid oxidase	"	"
Glycolic acid dehydrogenase	"	"
Lactic acid dehydrogenase	"	"
DPN ase	Neurospora	Nason et al. (41)
Decreased Activity		
Catalase	Tomato plant	Bean (4)
Dehydrogenase	"	Reed (47)
Tryptophan synthease	Neurospora	Nason (40)
Aldolase	Oats, Clover	Quinlan-Watson (44)

The study of Nason et al. is the most comprehensive yet reported on the effect of micronutrient deficiencies on enzymic constitution of higher plants. The effect of deficiencies of zinc, copper, manganese, iron, boron, molybdenum, and iron on several enzymes was tested. Except where the element in question was a part of the enzyme (e.g. copper in ascorbic acid oxidase), deficiency of micronutrients led to an increased activity of the enzymes tested. This increase in enzymic activity did not show any specificity for any particular deficiency. To account for this finding Nason et al. put forward the hypothesis that micronutrients are principally concerned with protein synthesis and are necessary for "key reactions". Where there is deficiency of micronutrient, these "key reactions" can not take place and the relatively "primitive" proteins that do not require the products of the "key reactions" increase in activity. This hypothesis is very plausible and accounts for all the experimental facts. Enzymes that are reduced in activity under conditions of micronutrient deficiency require the products of the postulated "key reactions", whereas the enzymes that increase in activity can be synthesized independently of these products.

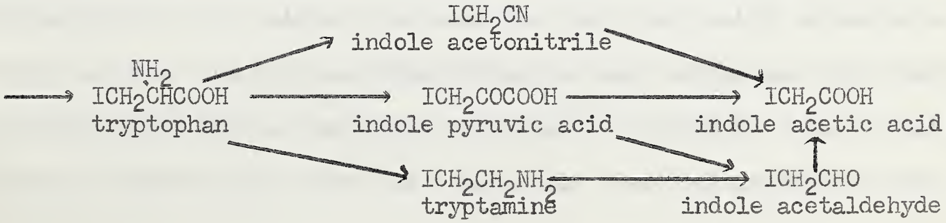
The metabolism of indoleacetic acid (IAA) Skoog (50), and Tsui (62) have found that zinc deficient tomato plants contain less auxin than healthy plants. This reduction in auxin content is detected before there are any morphological indications of zinc deficiency. It is now generally considered that the only natural auxin is IAA, and it is of interest to examine the effects of zinc deficiency on its metabolism.

The metabolism of IAA in normal plants is not fully elucidated, but a discussion of the present knowledge is a necessary introduction to a discussion of the effects of zinc deficiency on the enzyme systems involved. Workers on the precursors of IAA are agreed that tryptophan

is an essential precursor. There are differences about the products intermediate between tryptophan and indoleacetic acid, but these may be actual species differences rather than true disagreements.

Working with spinach plants as source material, Wildman, Ferri, and Bonner (69) concluded that tryptophan is first oxidatively deaminated forming indolepyruvic acid which is then oxidatively decarboxylated to form indole acetic acid. Gordon and Nieva (27), working with pineapple as source material, concluded that the immediate precursor of IAA is indoleacetaldehyde. They also found that both indolepyruvic acid and tryptamine were converted to indoleacetic acid by tissue extracts. Tryptamine could be formed from tryptophan by decarboxylation, and it could then be converted to indoleacetaldehyde by oxidative deamination. A third possible pathway is suggested by the discovery that indoleacetonitrile has growth regulating properties (5, 6). Thimann (61) considers that indoleacetonitrile only has growth regulating properties because of the ability of the plant to convert it to IAA. He demonstrated this in the following way: indoleacetonitrile is inactive in the pea test for auxins, but it is active in the *Avena* test. If the active molecule is the acid rather than the nitrile, then the difference between the two tissues must be in their ability to convert the nitrile into the acid. If this is true then *Avena* material should give rise to an active product in the pea test when it has been incubated with indoleacetonitrile. This is found to be the case.

A summary of the possible pathways between tryptophan and indoleacetic acid is given in the diagram.



The content of IAA is maintained in a dynamic manner, the concentration level is determined by synthesis on the one hand and destruction on the other. The enzyme system responsible for the destruction of IAA has come to be known as IAA oxidase, and has been intensively studied, mainly by Galston and co-workers (15 - 21).

It had been known for some time that oxidizing agents such as hydrogen peroxide could inactivate auxin. In the extraction of auxin for the *Avena* test, care had always to be taken to use peroxide free ether. The first report of enzymic inactivation of auxin came from Tang and Bonner (57). They found the enzyme in etiolated pea seedlings and described several characteristics. Ina second paper (58) they describe the physiology of the enzyme, and give an account of the presence of a naturally occurring inhibitor which seems to be more abundant in green tissues. The destruction of indoleacetic acid was found to be catalyzed by light in the presence of riboflavin (Galston, 15). This discovery was followed by a series of papers by Galston and co-workers on the physiology of light action (16, 17, 18, 19, 20). It was found that light inhibited auxin induced growth, this effect being concurrent with a destruction of the added auxin. (16). Although riboflavin stimulated growth in the dark, in the light its effect on growth was always inhibitory. It was also shown that the diffusible auxin of *Avena* coleoptiles is completely photoinactivated in the presence of riboflavin. Up to this point the photoinactivation of auxin investigated by Galston could be accounted for in terms of the purely physico-chemical system which he had first reported (15). Subsequently (17), Galston concluded that the inactivation of auxin in his tests was due to a flavin-enzyme, which was heat labile and inhibited by inorganic ions such as manganese and copper. As with the preparation of Tang and Bonner (58), there was a naturally occurring inhibition which

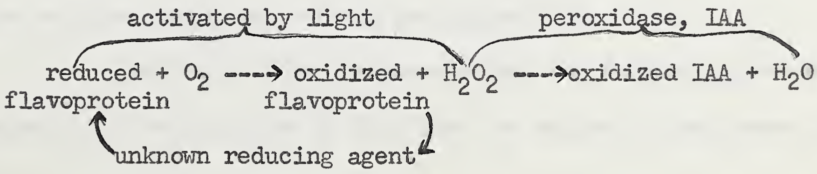
could be removed by dialysis or reversed by illumination. The dialysed preparations were not activated by light. It was found that manganese ions had all the properties of the naturally occurring inhibitor; however, the action of manganese ions has been shown to be stimulatory in other cases (Wagenknecht and Burris, 67; Gortner and Kent, 29). The action of these ions is not yet fully understood.

Further studies by Galston have shown that the metabolism of indoleacetic acid is even more complicated than might first appear. For example the auxin induced growth of green pea seedling sections (as opposed to etiolated pea seedlings) is stimulated by light. In this response the stimulation by light can be replaced by supplying sucrose (19). Further, the inhibition of growth in auxin in the presence of light is found with extremely small quantities of light, whereas the light enhancement of auxin induced growth is increased with increasing exposure to light; no limit was reached even after 24 hours. Because of this tremendous difference in the effect of auxin induced growth in green and etiolated tissues, it is difficult to evaluate the findings with etiolated tissue and apply them to an understanding of auxin metabolism in the normal plant. In spite of this reservation, Galston and Dalberg (22) have obtained results on the adaptive formation of IAA oxidase in etiolated pea seedlings which they used to form very satisfactory hypotheses to account for aging, lateral bud inhibition, and interactions of auxins. They found that older tissues had greater IAA oxidase activity which they considered to be induced by the polar transport of auxin from the meristematic regions to the older tissue. Increased activity of the enzyme with increasing age was shown to be a response to elevated auxin levels. The enzyme is present only in small quantities in actively growing cells.

Wagenknecht and Burris (67) found IAA oxidase in bean roots

and in pea seedlings. While manganese was stimulatory in the case of the enzyme from bean roots, the effect was variable in the case of the enzyme from pea seedlings, frequently causing inhibition. In both cases, however, high concentrations of manganese ions were inhibitory. Wagenknecht and Burris suggest that the optimum level of manganese is already present in the pea extract, whereas the bean root preparation requires supplementation with respect to manganese. The enzyme has also been found in pineapple tissue by Gortner and Kent (29). This enzyme was stimulated by manganese ions, but in other respects it differed from the enzyme from other tissues reported above. Its pH optimum is approximately 3.5 whereas the pH optimum for the enzyme from other sources is about 6.5. In contrast to the enzyme from pea seedlings, the pineapple enzyme is not stimulated by dichlorophenol; and its activity is not increased by dialysis. It therefore appears that though the enzyme from pineapple accomplishes the same oxidation, it is not the same as the enzyme from pea seedlings and bean roots.

Examinations of the protein nature on indoleacetic acid oxidase had indicated that it had a metallo-enzyme component (57, 67) and also that a second, light activatable component was of flavin nature. These discoveries were integrated in a paper of Galston, Bonner, and Baker (21). Their work shows that IAA oxidase rather than being a single enzyme is an enzyme system composed of a flavoprotein which is responsible for the production of hydrogen peroxide, and a peroxidase component which utilizes the hydrogen peroxide for the destruction of indoleacetic acid. The scheme suggested is shown below:


activated by light
peroxidase, IAA
reduced flavoprotein + O_2 ----> oxidized flavoprotein + H_2O_2 ---->oxidized IAA + H_2O
unknown reducing agent

The inhibition of IAA oxidase by catalase can be accounted for as a removal of hydrogen peroxide which might otherwise be used in the oxidation of indoleacetic acid. Similarly the inhibition by manganese can be interpreted also as a removal of hydrogen peroxide since Kenten and Mann (34) have found that there is a system in horse radish root extracts which oxidizes manganese in the presence of hydrogen peroxide. Andreae and Andreae (3) explain the inhibition of IAA oxidase by scopoletin as a preferential use of the available hydrogen peroxide for the destruction of scopoletin rather than IAA. They are also able to support the suggestion of Galston et al. (21) that IAA itself is the unknown reducing agent which gives rise to hydrogen peroxide by virtue of the discovery that IAA stimulates the oxidation of scopoletin, presumably by increasing the supply of hydrogen peroxide. IAA oxidase is known to be stimulated by 2,4-dichlorophenol (DCP) (Goldacre, Galston, and Weintraub, 25). Though this substituted phenol is known to inhibit catalase (Goldacre, and Galston, 24), it has been concluded that stimulation of IAA oxidase is by some other mechanism, since a catalase free IAA oxidase preparation was still stimulated by DCP. Lockhart (38) concludes that DCP is effective in the peroxidase portion of IAA oxidase, whereas Siegel and Galston (49) conclude that DCP is effective in the peroxide genesis step, i.e. the flavoprotein component. The genesis of peroxide is clearly of importance in the destruction of IAA. Increased concentration of peroxide may be expected to result in greater destruction of IAA. The report of Galston and Siegel (23), that manganese stimulated the genesis of peroxide would suggest that manganese would stimulate IAA destruction as was the case with bean roots (67), but is not in agreement with the earlier report of Galston and Baker (18).

The work on IAA oxidase leaves the following points unclear: the mechanism of stimulation by DCP, and the action of manganese ions. In

both cases the effects have yet to be related to reactions of natural physiological importance. To these outstanding problems may now be added another recently brought up by Goldacre (26). He has found that there is photochemical inactivation of IAA sensitized by non-protein components of plant tissue. These components are found in the non-protein dialysate of the brei of etiolated pea epicotyls. Goldacre states that chromatographic analysis indicates that there are at least four active components. It is possible that these components photoinactivate IAA in the same way as riboflavin (15).

The above review summarizes the discoveries so far made on the metabolism of IAA in higher plants. However, it will be shown later that the synthesis of tryptophan, a precursor of IAA, is of great importance in the study of zinc deficiency in higher plants. For this reason the synthesis of tryptophan will be discussed even though the mechanism for higher plants has not yet been reported.

Tatum and Bonner (59) have reported the synthesis of tryptophan from indole and serine by Neurospora. Indole alone disappeared slowly from the medium; the stimulation of indole uptake was accomplished by adding serine, whose stimulation was highly specific, l-serine being twice as effective as dl-serine. Yields of tryptophan were about 40% of the theoretical maximum, the remainder apparently had been further metabolised, one of the products being IAA. The synthesis of tryptophan by a similar reaction has been reported to occur in Lactobacillus arabinosus. (Schweigt, Sauberlich, Baumann, and Elvehjem, (48)).

Later Umbreit, Wood and Gunsalus (65) prepared a cell free extract from Neurospora which was active in the synthesis of tryptophan from indole and serine. It was found that pyridoxal phosphate was a necessary co-factor of the reaction. Stored preparations lost activity, but the activity could be restored by the addition of the co-enzyme.

From the point of view of zinc deficiency, the metabolism of IAA will be divided into three sections: the synthesis of tryptophan, the conversion of tryptophan to IAA, and the oxidation of IAA.

i) In the synthesis of tryptophan by Neurospora, Nason (40) and Nason et al. (41) found that zinc deficiency affected the condensation of indole and serine unfavorably. Deficiencies of iron and manganese did not affect the activity. Addition of zinc solutions did not affect the activity of the enzyme. McElroy and Nason (39) have suggested that zinc deficiency might affect the same reaction in tomato plants and so account for the reduced content of tryptophan and indirectly for the reduced content of IAA.

ii) The diversities in the conversion of tryptophan to IAA can fortunately be neglected in the study of zinc deficiency. Tsui (62) incubated leaf disks of deficient and control plants in solutions of tryptophan and subsequently assayed for IAA. He found that the disks from deficient plants had ability to convert tryptophan to IAA equal to that of the healthy plants. Wiltshire (71) has found that in pea seedling tissue and extracts there is an enzyme which peroxidatively destroys tryptophan. Even though peroxidase is more active in zinc deficient plants, it appears that tryptophan is not affected in zinc deficient tomato plants.

iii) The knowledge that tryptophan content is reduced in zinc deficient plants has placed emphasis on the synthesis of IAA as the system affected by zinc deficiency. But there is reason to believe that the early ideas of Skoog (50) on the destruction of auxin may be applicable. Skoog found that when stem sections of zinc deficient and healthy plants were placed on agar blocks containing known amounts of IAA, there was greater destruction by the deficient sections. However, he was not able to show this destruction in cell free extracts. Since this work of Skoog, the enzyme IAA oxidase has been discovered, and there are a number of

points of similarity in the physiological effects of zinc deficiency and the characteristics of the enzyme.

Zinc deficiency symptoms are more acute under high light intensity. Peroxidase activity is increased, and catalase activity is decreased. High light intensity similarly stimulates IAA oxidase, and since the enzyme is a peroxidase, more IAA would be oxidized under zinc deficiency. Catalase inhibits IAA oxidase, and therefore zinc deficiency might be expected to increase the activity of IAA oxidase in this case also.

This circumstantial evidence indicates the IAA oxidase is more active in zinc deficient plants. The work of Skoog therefore should be substantiated and extended.

MATERIALS AND METHODS

Plant Material

Tomato plants, *Lycopersicon esculentum* (variety Bounty), were used in all experiments.

Seeds were soaked overnight in distilled water. They were then germinated in moist vermiculite. After about one week, when the seedlings were 1½ - 2 inches in height, transplanting was carried out. Culture of the plants was essentially as described by Stout and Arnon (54). The plants were supported in a perforated disk made of plaster of Paris and coated with paraffin wax. The roots hung down into the culture solution, which was held in 2-liter pyrex beakers. Macronutrient solutions were prepared and purified by the precipitation of impurities with calcium carbonate as described by Stout and Arnon (54), the theoretical considerations of which are discussed by Steinberg (52). Micronutrients were supplied as the A4 solution of Arnon (1). Iron was supplied as ferric tartarate, 1 ml. of a 0.5 % solution being added to each beaker twice a week.

Macronutrient solutions and the water used in the beakers were tested for zinc content by the dithizone method (54). Demineralized water was found to contain less zinc than glass redistilled water and was used throughout. Final concentrations of zinc in the culture solutions were:

Control - 0.05 parts per million

Zinc deficient - less than 0.0005 parts per million

The beakers were set in the greenhouse as indicated in Figure 1 a. Entry of light and consequent algal growth was prevented by wrapping the beakers in brown wrapping paper. The period of growth was up to six weeks; during this time the level of the solution was maintained

by additions of demineralized water.

Tissue slices were cut freehand with a razor blade and washed with distilled water before use. Leaf disks were cut from the leaves with a cork borer and were washed with distilled water before use. Some tissue extracts were prepared by homogenizing in the Waring blendor. Because of the heating and aeration involved, and because of the detrimental effects described by Stern and Bird (53), this method was discarded. Extracts of small samples were prepared in a glass homogenizer of the Potter-Elvehjem type. For larger samples, the method generally followed was to grind the tissue with an equal weight of demineralized water and a small amount of acid washed sand in a mortar. The resultant brei was pressed through cheesecloth, and the liquid was centrifuged at 3,000 r.p.m. for 30 minutes to remove coarse fragments.

Enzymes

Polyphenol oxidase activity was determined by following oxygen uptake in the Warburg apparatus. Catechol was used as substrate, being added from the side arm at zero time.

Tryptophan synthease activity was measured by a modification of the method of Nason, Kaplan and Colowick (41). The assay was performed in 50 ml. erlenmeyers containing 10 ml. buffer, indole, serine, enzyme, and distilled water to make up the volume to 20 ml. The vessels were stoppered with cotton wool and shaken at room temperature. At intervals, 2 ml. of the reaction mixture were taken and shaken with toluene to extract the residual indole. An aliquot of the toluene layer was taken and analysed for indole concentration. An aliquot of the aqueous layer was analysed for tryptophan concentration.

The conversion of tryptophan to IAA was measured in 50 ml. erlenmeyers which contained tissue or tissue extract, tryptophan, and

buffer, in a total volume of 20 ml. The flasks were stoppered with cotton wool and shaken at room temperature. Samples of the reaction mixture were taken at intervals for determination of IAA concentration.

Two methods were employed in the estimation of IAA oxidase:
a) the method of Tang and Bonner (57). Enzyme, buffer, substrate and addenda were incubated in 20 ml. volume under the same conditions as described for tryptophan synthease. Samples were taken at intervals for determination of IAA concentration.
b) the manometric method of Wagenknecht and Burris (67). The only modification made was that the IAA was placed in the main compartment instead of being added from the side arm at zero time.

Analytical Methods

Tryptophan was determined by a modification of the method of Horn and Jones (31). The aliquot to be tested was placed in a colorimeter tube and made up to 1 ml. with distilled water. One ml. of a 5% solution of p-dimethylaminobenzaldehyde in concentrated HCl was added followed by 3 ml. of concentrated HCl. The addition of sodium nitrite, as described by Horn and Jones, was not found to benefit color development, and so was excluded. The color density was read in a colorimeter after 30 minutes at a wavelength of 550 millimicrons. Concentration was then calculated by reference to a standard curve relating color density to concentration.

Determination of indole was by the method of Wood, Gunsalus and Umbreit (73), the only modification being that the test solution was made up to 5 ml. with acid alcohol instead of 10 ml.

IAA was determined by the method of Tang and Bonner (57) except that, for convenience, 5 ml. instead of 4 ml. of the Salkowski reagent were added to the test aliquot.

Protein nitrogen was determined by a modification of the method of Galston and Dalberg (22). One ml. of the tissue extract was placed in a plastic centrifuge tube and one ml. of 1.0 M trichloracetic acid was added. After standing in the cold room for at least 30 minutes, the precipitate was centrifuged down at 3,000 r.p.m. for 30 minutes, and was then resuspended in 0.5 M trichloracetic acid. The precipitate was again centrifuged down, and the supernatant poured off. The precipitate was now transferred to a digestion flask with the aid of several washings of demineralized water, and 1 ml. of the digestion mixture added. This digestion mixture was prepared by adding 500 mgm. of mercuric sulfate and 50 mgm. of sodium selenite to 200 ml. of concentrated sulfuric acid, and making the volume up to 1 liter with demineralized water. Digestion was aided by cooling after 30 minutes heating, and adding two drops of Superoxol. After further heating, this step was repeated. The contents of the digestion flask were then cooled and made up to a volume of 25 ml. An aliquot of this solution was taken and made up to 5 ml. in a colorimeter tube. One ml. of Nessler solution was added and the color read at 425 millimicrons after 15 minutes. (The Nessler solution was prepared by mixing one part Eimer and Amend Folin-Wu Nessler reagent and four parts 10% NaOH). Nitrogen was calculated by reference to a standard curve. Determinations were carried out in duplicate, and corrections were made from the reading of a reagent blank.

Dry Weight

Samples were dried at 60 degrees C. for several hours, and then at 110 degrees C. for about one hour before being cooled in a dessicator and weighed.

Manometric Measurements

Oxygen uptake was measured in the Warburg apparatus in an atmosphere of air at 28 degrees C. The total volume in each vessel was 2.2 ml. KOH was present in the center well.

RESULTS

I. Morphological

The superior growth of the control plants was usually visible after 2 - 3 weeks growth in the culture solutions. However, it was noticeable that during the months October - February, deficiency symptoms did not appear at any time. The solutions were prepared from the same stock solutions that had given rise to deficiency symptoms previously, and which gave rise to deficiency symptoms subsequently.

Plants having an inadequate supply of zinc had a generally stunted growth habit, as compared to the normal plants (Figure 1, b). The internodes were short. The petioles were reflexed. The leaves were malformed, and later developed necrotic spots; finally they withered completely (Figure 1, c, d). Root growth was considerably less under conditions of zinc deficiency.

Recovery from these effects could be obtained by the addition of zinc sulfate to bring the concentration of zinc to that in the control beakers (Figure 1, e, f).

II. Uptake of Indole by Tomato Tissues and Tissue Extracts

The uptake of indole was measured by the method described under Materials and Methods. Materials used included cotyledons, leaf disks, stem slices, and extracts of these tissues. No previous report of the utilization of indole by higher plants had been seen in the literature, so the enzyme responsible for the uptake of indole was first characterized.

Effect of pH Preliminary experiments with cotyledons indicated that the optimum pH for the uptake of indole was about 5.0. The same optimum was obtained when the experiments were performed with leaf extracts. The shape of the pH-activity curve is shown in Figure 2.

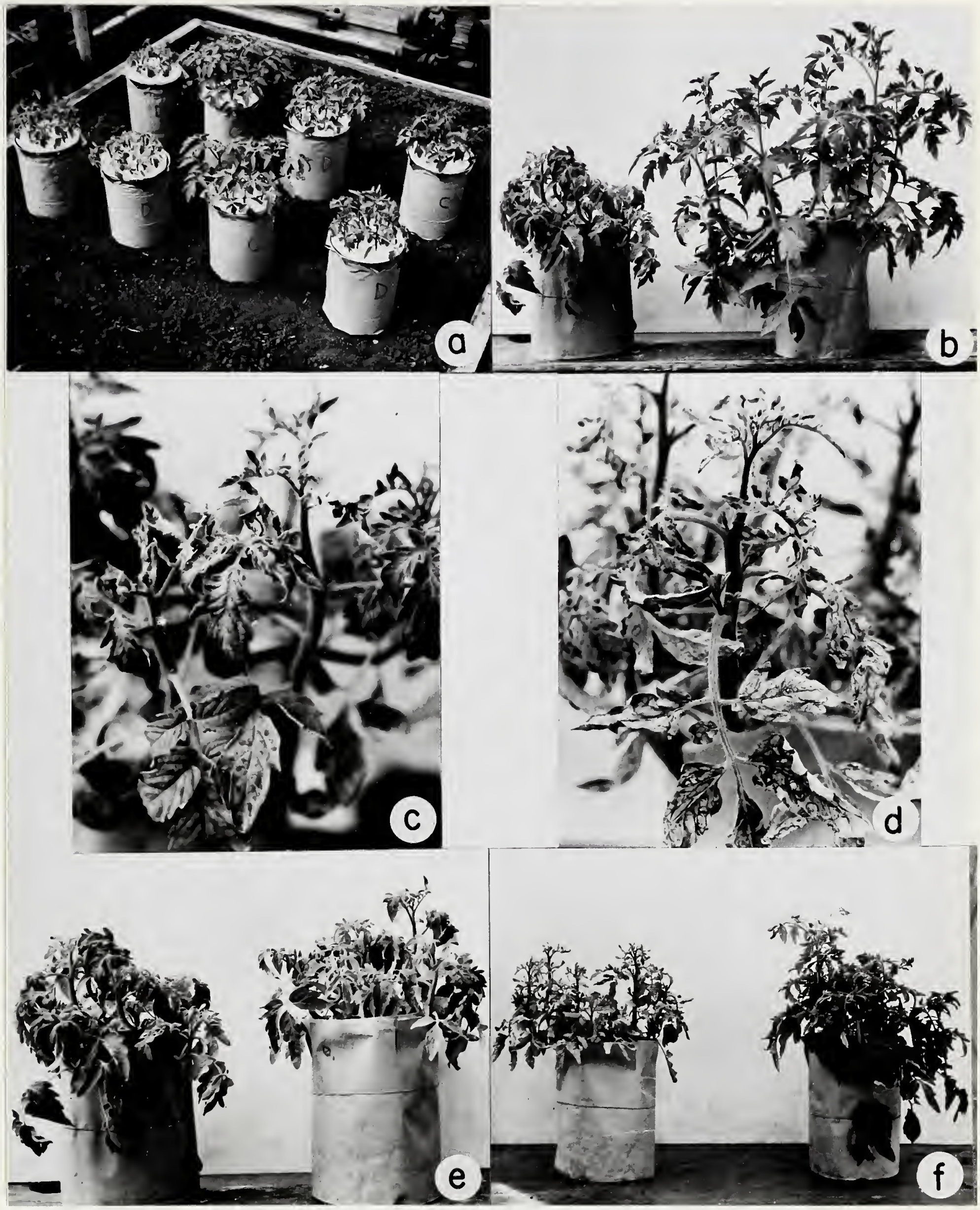
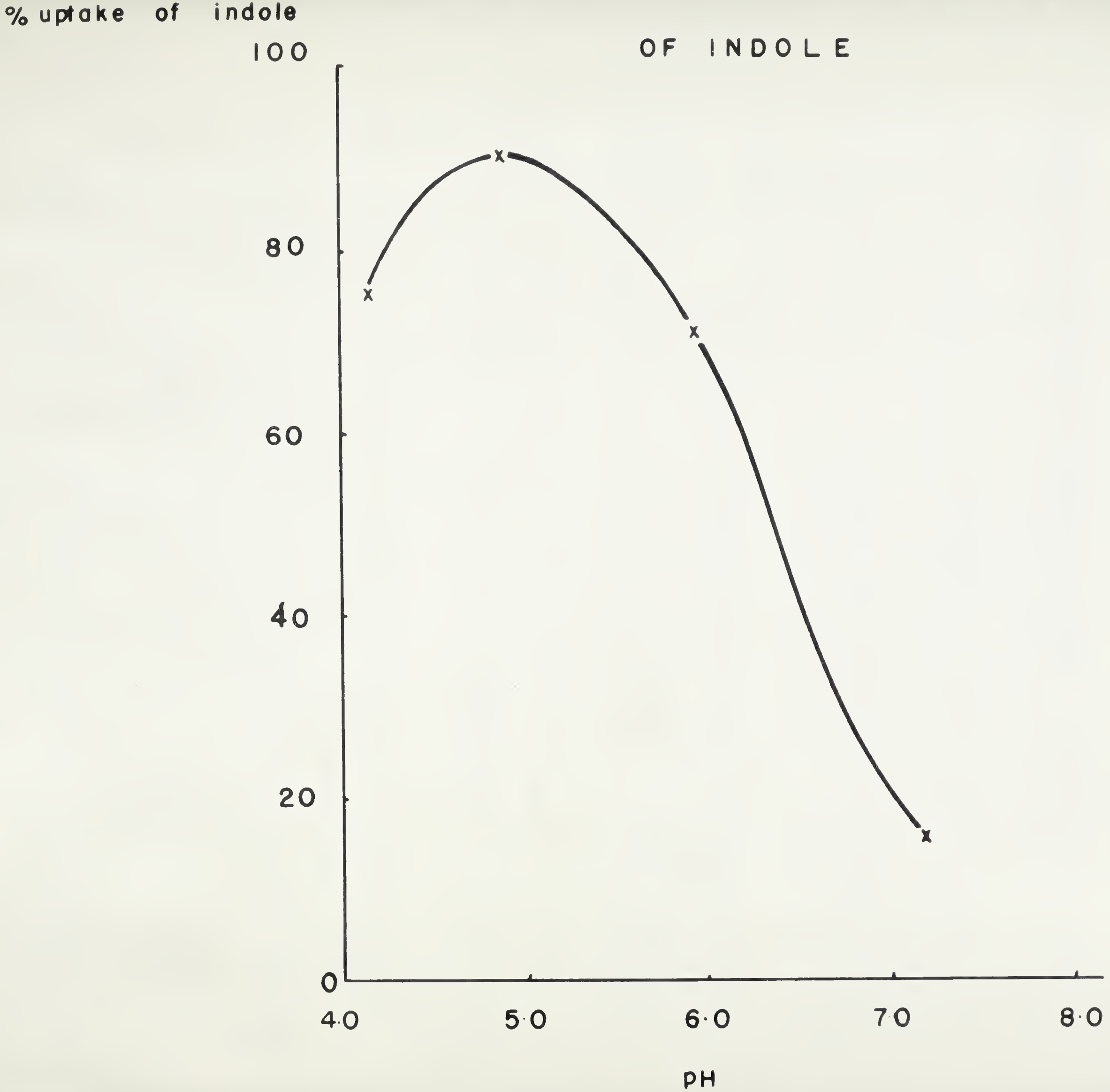


Figure 1. a) Experimental layout.
b) Zinc deficient and control plants at same age.
c) Early stages of zinc deficiency.
d) Deficiency symptoms 2 weeks later than c).
e) & f) Recovery from zinc deficiency; zinc sulfate solution was added to the beaker on the right.
f) shows the amount of recovery in 2 weeks.

FIGURE 2. INFLUENCE OF pH ON UPTAKE OF INDOLE



The reaction mixture contained 10 ml. phosphate-citrate buffer of the indicated pH; 4 ml. of the tissue extract; 3 ml. 10^{-3} M indole; 2 ml. 10^{-2} M dl-serine; and 1 ml. distilled water. Samples were taken for analyses at zero time and after two hours incubation.

The optimum pH is much lower than pHs which are considered to be physiological, but the same result was obtained consistently.

Progress of the Reaction in Relation to Time In some of the earlier experiments long periods of incubation were allowed. It was later found that such long periods were unnecessary for tissue extracts because the reaction was virtually complete after a period of two hours. With whole tissue the progress of the reaction was slower presumably because of the limitation of diffusion of substrate into the cells. Time-progress graphs are shown in Figure 3.

The reaction mixture contained 10 ml. of phosphate-citrate buffer, 5 ml. of the leaf tissue extract, 3 ml. 10^{-3} M indole, and 2 ml. 10^{-2} M dl-serine. Samples were taken for analyses at the indicated times.

Effect of Serine Concentration If the uptake of indole is catalyzed by an enzyme system similar to the one found in Neurospora, then there would be formation of tryptophan, and a requirement for serine. As will be mentioned later the colorimetric test for tryptophan was not adequate even for the estimation of theoretically maximum quantities formed. The requirement for serine however would be strong evidence that the enzyme is the same as the one found in Neurospora (59).

Experiments with tomato cotyledons showed that increasing concentration of dl-serine stimulated the uptake of indole up to a point, but there was reduced uptake at higher concentrations. The results of one experiment are shown in Figure 4. In a duplicate experiment, essentially the same results were obtained.

FIGURE 3. TIME - PROGRESS OF INDOLE UPTAKE

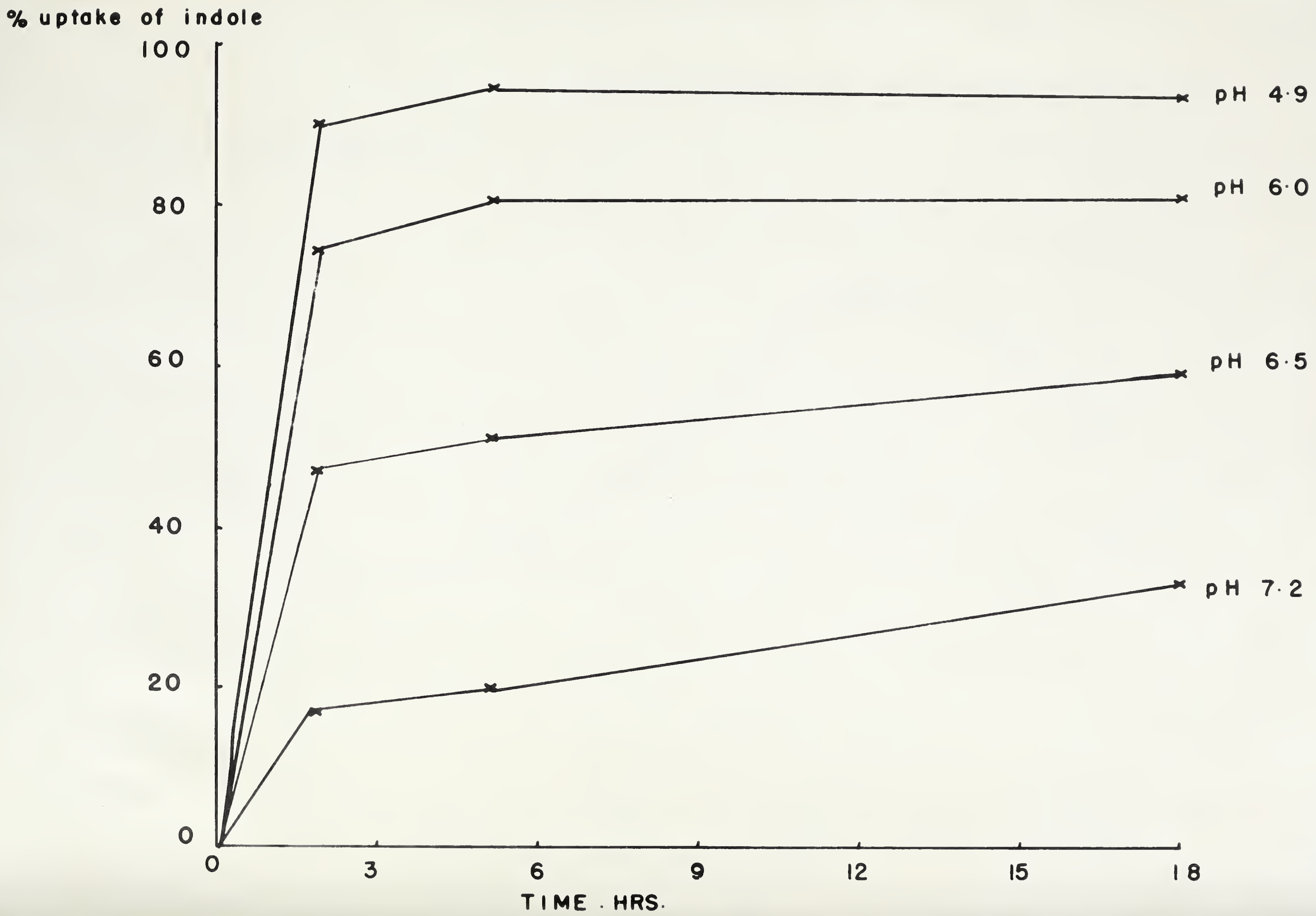
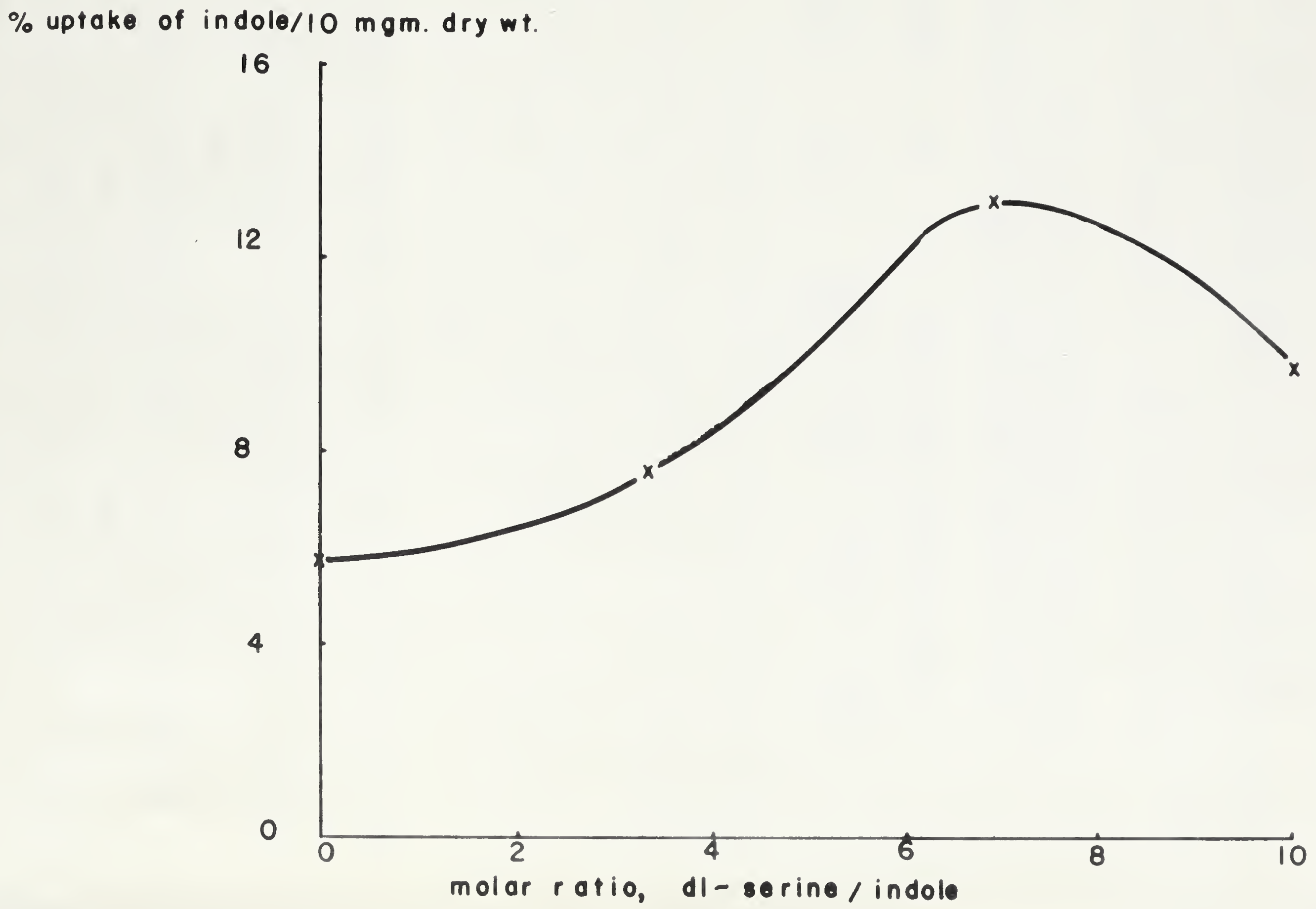


FIGURE 4. SERINE DEPENDENCY OF INDOLE UPTAKE



The reaction mixture contained 10 ml. phosphate citrate buffer, pH 4.9; 3 ml. 10^{-3}M indole; varying amounts of 10^{-2}M dl-serine; and distilled water to make the volume up to 20 ml. The dry weights of the cotyledons were determined at the end of the experiment. Samples for analyses were taken at zero time and after 8 hours incubation.

With tissue extracts there was no indication that serine is necessary for the uptake of indole. Addition of dl-serine either had no effect or caused some inhibition, as shown in the following table.

Table 2. Effect of Serine Concentration on Uptake of Indole

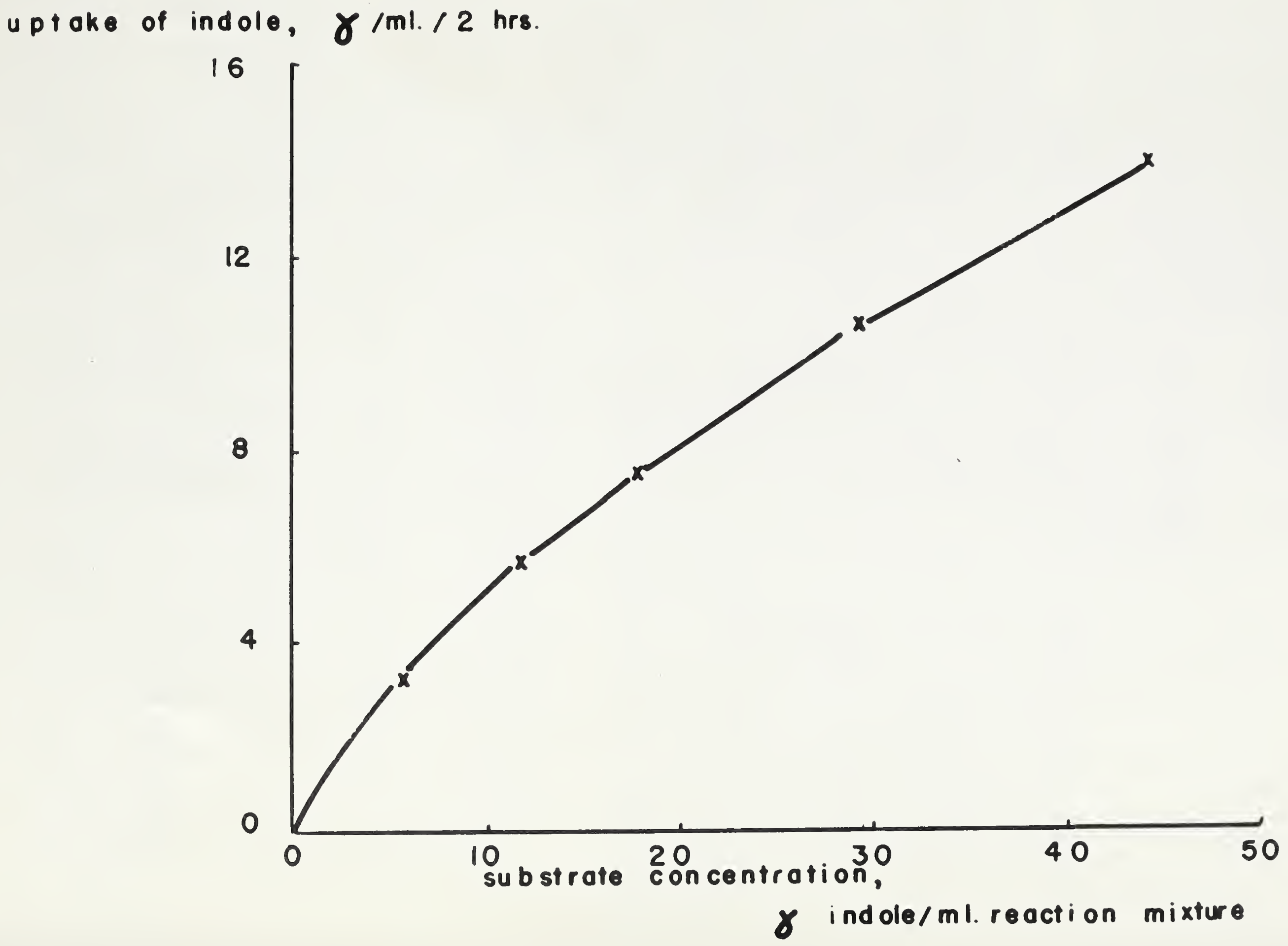
(Micrograms/ml. reaction mixture/mgm. protein nitrogen)

Material	pH.	Molar ratio, dl-serine/indole			
		0	3.3	6.6	10.0
leaf	6.0	252	254	205	229
stem	6.5	100	85	95	66

The absence of stimulation of the tissue extracts by added serine may be due to the release of serine from the tissues during the preparation of the extract. Inhibition may be due to enzyme specificity for the l-isomer. To test this idea it will be necessary to purify the enzyme by precipitation techniques so that there can be no contamination by serine. This has yet to be done.

Effect of Indole Concentration According to the theory of Michaelis and Menten as described by Wilson (70), the effect of substrate concentration on reaction velocity should be described by the equation of a rectangular hyperbola. The concentration of indole was indeed found to affect the uptake of indole in this manner, as shown in Figure 5. Lineweaver and Burk (37) have shown that agreement with the theory of Michaelis and Menten can be better demonstrated if the data are manipulated in order to obtain a straight line. This can be done by plotting I/V against I/S.

FIGURE 5 EFFECT OF SUBSTRATE CONCENTRATION



Such a plot is shown in Figure 6. The theoretical considerations of this method are as follows:

The Michaelis-Menten equation is commonly written:

$v = V(S)/(Ks + (S))$, where:

v is the initial reaction velocity,

V is the maximum velocity,

(S) is the concentration of the substrate,

Ks is the Michaelis constant.

This Michaelis constant is the dissociation constant of the enzyme-substrate complex, and is often defined as the substrate concentration which gives half maximal velocity of reaction. When substrate concentration is plotted directly against velocity it is frequently difficult to evaluate the Michaelis constant because the maximum velocity is not reached under the experimental conditions. This can be seen to be the case in the effect of indole concentration on indole uptake. The method of Lineweaver and Burk removes this difficulty, the maximal velocity being found by extrapolation. The Michaelis-Menten equation is rearranged:

$I/v = Ks/V(S) + I/V$

When I/v is plotted against I/(S), a straight line is obtained with an ordinate intercept I/V and a slope of Ks/V. From these figures Ks can be calculated.

From Figure 6, the following values were obtained:

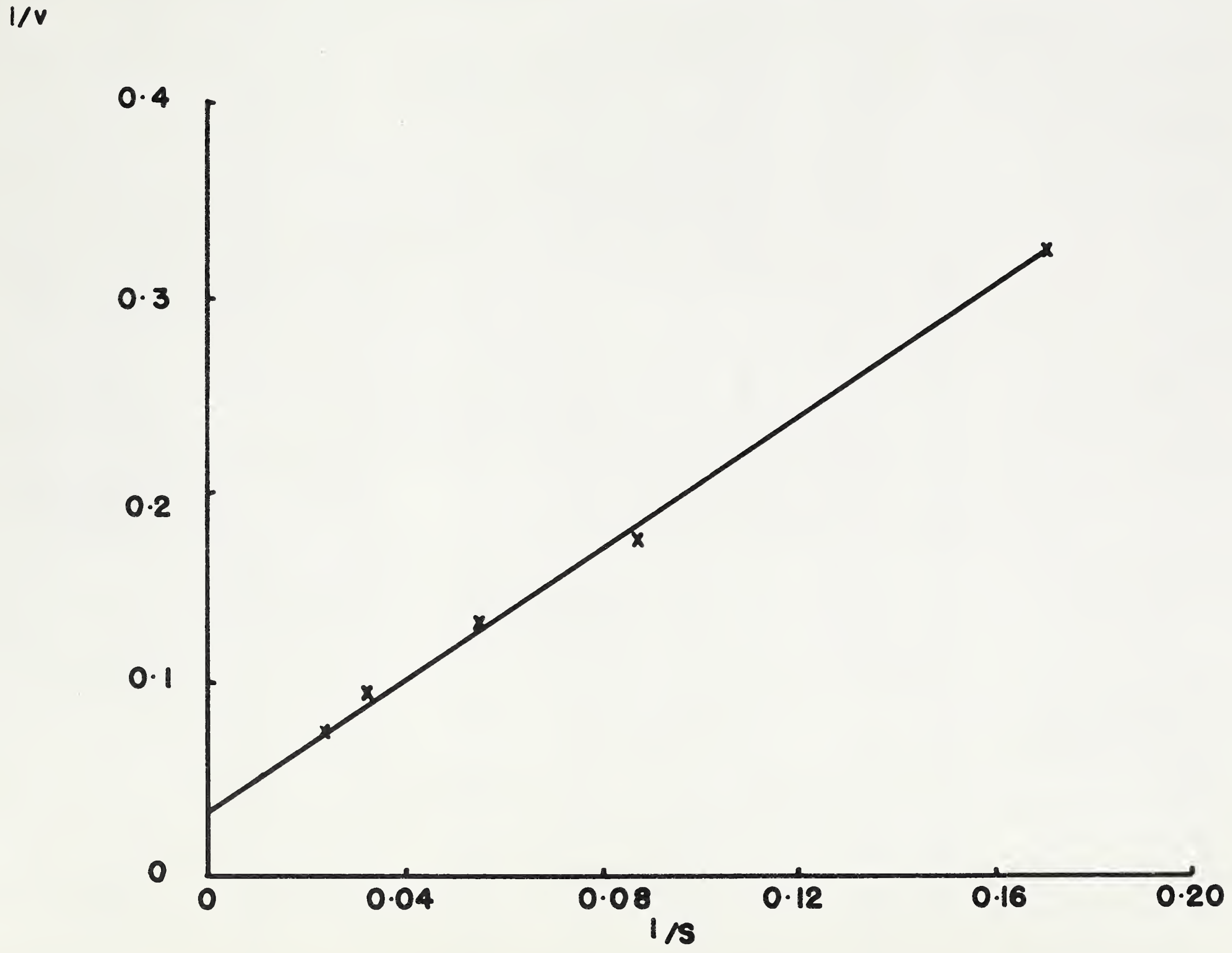
Ks, 4.0×10^{-4} M

V, 28 micrograms indole taken up / ml. / 2 hours.

Repetition of the experiment gave almost identical results.

The reaction mixture contained 10 ml. phosphate-citrate buffer pH 6.5; 2 ml. enzyme preparation; varying amounts of 2.5×10^{-3} M indole (293 γ indole/ml.); and distilled water to make the volume up to

FIGURE 6. EFFECT OF SUBSTRATE CONCENTRATION, LINEWEAVER - BURK PLOT



20 ml. The supply of serine was considered to be adequate in view of the findings described in the previous section.

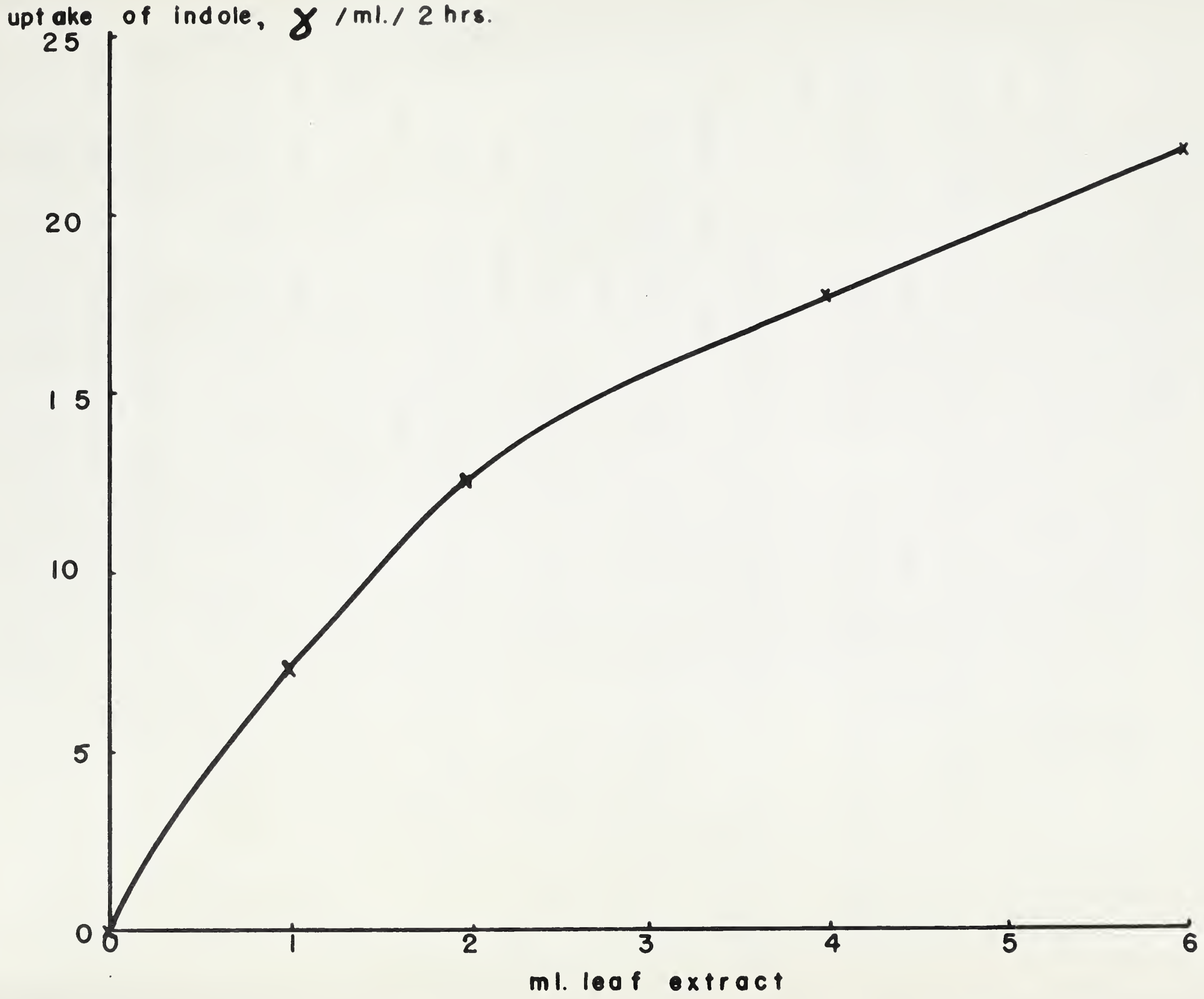
Effect of Enzyme Concentration The effect of enzyme concentration on the uptake of indole by tomato leaf extract is shown in figure 7. Sumner and Somers (56) state that the reaction velocity is usually directly proportional to the enzyme concentration provided that an excess of substrate is present. The graph in this case departs from linearity, particularly at higher concentrations of enzyme. This may be attributed to insufficient excess of indole.

The reaction mixture contained 10 ml. phosphate-citrate buffer, pH 6.5; 3 ml. $2.5\text{x}10^{-3}$ M indole; varying amounts of tissue extract; and distilled water to make the volume up to 20 ml.

Products of the Reaction Enzyme activity was usually measured as uptake of indole. However, in relation to zinc deficiency, the products of the reaction are of great importance. The experiments described above were carried out with the basic hypothesis that in higher plants indole is condensed with serine to form tryptophan. This hypothesis could only be verified by the identification of reaction products when indole disappeared from reaction mixtures.

Difficulty was experienced in the quantitative estimation of tryptophan as a product of the reaction. This was because the colorimetric test for tryptophan is much less chromogenic than the colorimetric test for indole. For example, 0.1 micromole of indole in solution increased the optical density of the Erlich test solution by 0.620. The same molar quantity of tryptophan increased the optical density by 0.055. Thus even when the disappearance of indole was marked, the formation of tryptophan did not cause a marked increase in optical density when the

FIGURE 7. EFFECT OF ENZYME CONCENTRATION

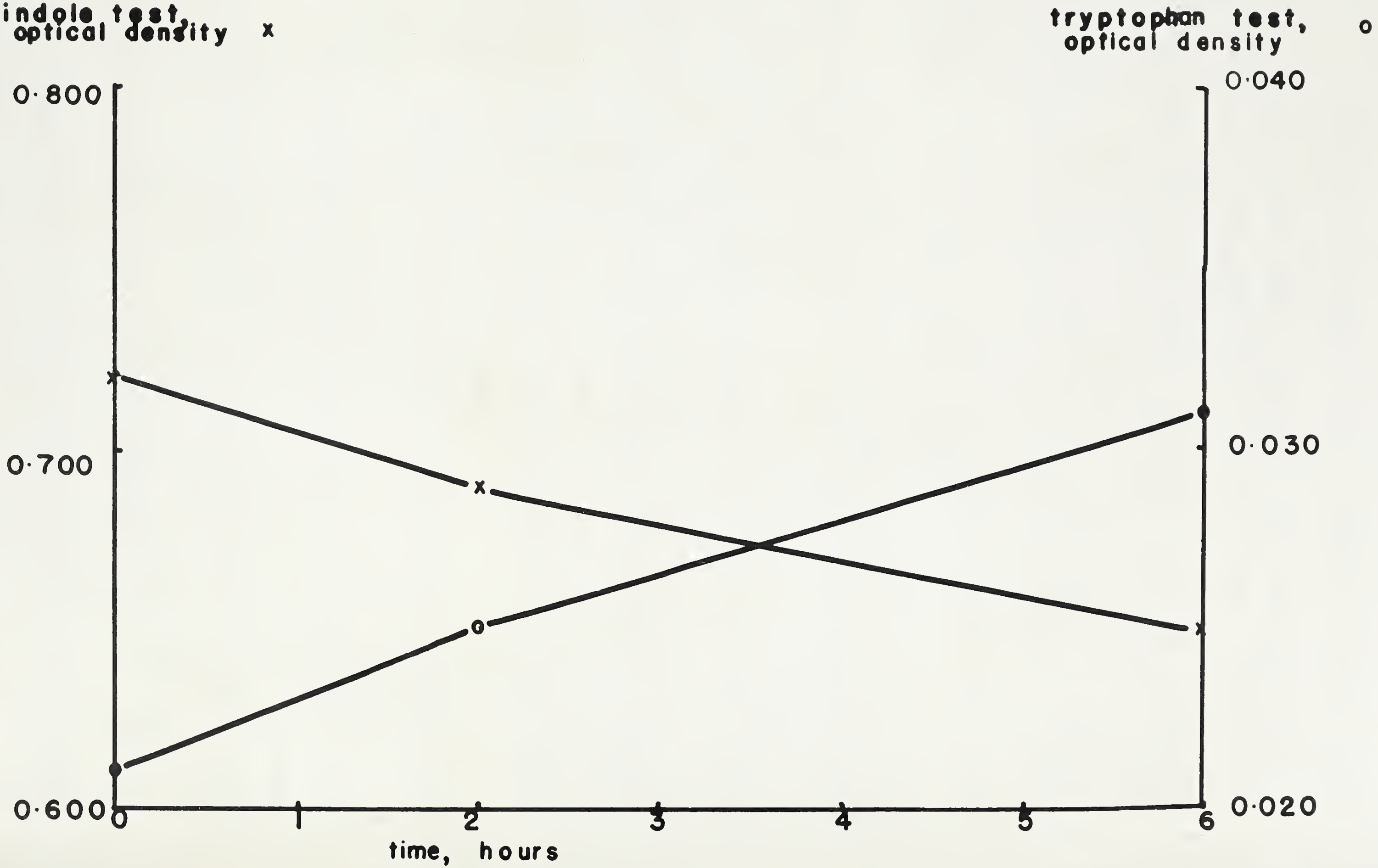


colorimetric test was applied. Nevertheless, experiments of a qualitative nature have shown that tryptophan is a product of the reaction involving the uptake of indole. The relationship of the formation of tryptophan and the disappearance of indole is shown by the optical density data graphically presented in Figure 8.

The reaction mixture contained 10 ml. phosphate-citrate buffer pH 4.9; 2 ml. 10^{-3} M indole; 2 ml. 10^{-2} M dl-serine; and distilled water to make the volume up to 20 ml. The active material was provided by tomato cotyledons. In another vessel serine was excluded; in this vessel the optical density of the tryptophan test solution stayed relatively constant. Other reaction vessels included a check for turbidity changes.

Because the production of tryptophan could not be put on a quantitative basis, some other method of measuring products was looked for. It is known that tryptophan gives rise to IAA (Wildman, Ferri, and Bonner, 69). Therefore formation of IAA from indole would provide presumptive evidence for the formation of tryptophan. Two problems arose here: the pH optimum for the uptake of indole has been shown to be about 5.0, whereas the pH optimum for the conversion of tryptophan to IAA is reported to be about 7.5 (Wildman, Ferri, and Bonner, 69). For the conversion of indole to IAA a pH between these had to be chosen. The second problem was the separation of indole and IAA for colorimetric analysis. Fortunately this was found to be possible by the same method as used for the separation of tryptophan and indole. Shaking an aqueous solution of the two compounds with toluene separates the indole into the toluene layer, and the IAA remains in the aqueous layer. The two compounds can not be measured by tests on the same solution because indole is chromogenic in the Salkowski test for IAA (Gordon and Weber, 28).

FIGURE 8. CONVERSION OF INDOLE TO TRYPTOPHAN



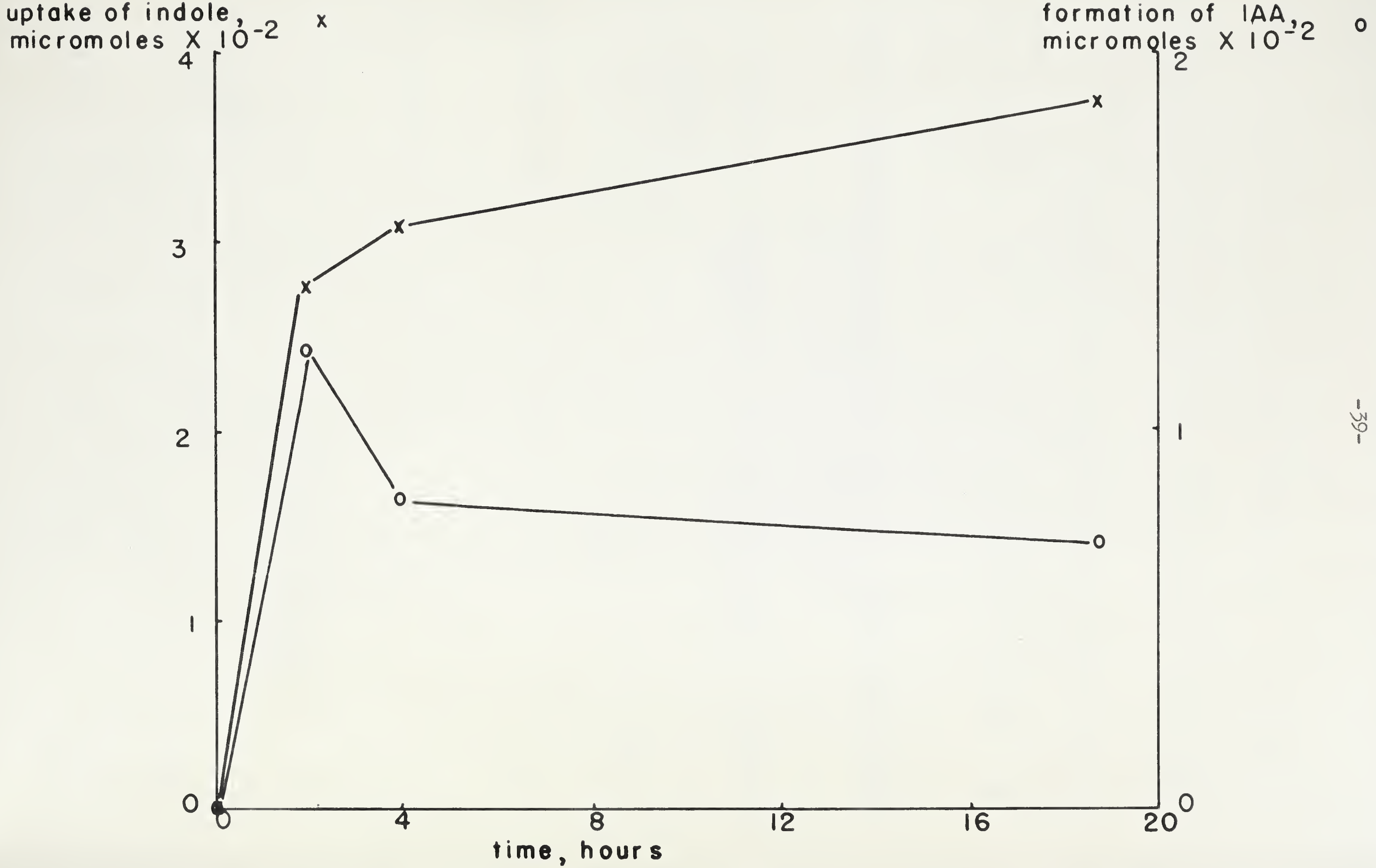
Conversion of indole to IAA in relation to time is shown in Figure 9. The reaction mixture contained 10 ml. phosphate-citrate buffer, pH 7.1; 3 ml. leaf extract; 2 ml. 10^{-3} M indole; and distilled water to make the volume up to 20 ml. Two other vessels were shaken at the same time; one contained the substrate with boiled enzyme, and the other contained the enzyme preparation with no substrate. Colorimetric tests on the solution in the latter vessel made it possible to correct the values in the experimental vessel for changes in turbidity. There was no activity in the boiled enzyme control.

The results of this experiment indicate that IAA is formed from indole, though it appears that IAA was destroyed by the system after it had been formed from indole. Even at the time when the concentration of IAA was at a maximum, (after 2 hours), it was only 43% of the theoretical maximum yield of the indole consumed. If this destruction of IAA is a feature of the tissue extracts, measurement of IAA formed is clearly unreliable as an estimation of the activity of the enzyme catalyzing the condensation of indole and serine. Other experiments have given yields of IAA closer to the theoretical maximum.

Work done on the products of the reaction consuming indole therefore show that both tryptophan and IAA are formed. No satisfactory method of measuring these products quantitatively has been found, though it may be said that the modification of technique so that tryptophan may be quantitatively estimated would be preferable because the estimation of IAA introduces several other enzymes besides the one of study. Such introductions reduce the control of the experiment.

Comparison of Zinc Deficient and Healthy Tomato Plants in Their Ability to Utilize Indole Having characterized the enzyme responsible for the uptake of indole, it was then undertaken to test the possibility that the

FIGURE 9. CONVERSION OF INDOLE TO IAA



enzyme might be affected by zinc deficiency. It has already been speculated that the activity of the enzyme condensing indole and serine might be reduced in activity in zinc deficient plants, so reducing the content of tryptophan directly, and the content of IAA indirectly (McElroy and Nason, 39).

Comparison of zinc deficient and healthy plant tissues and extracts, contrary to what was suspected, invariably showed the zinc deficient material to be more efficient in the utilization of indole. The results of several experiments are shown in Table 3.

Table 3. Comparison of Zinc Deficient and Healthy Tomato Plant Tissues in Their Ability to Take up Indole

Material		Time Incubated (hours)	pH	γ Uptake of Indole/Ml. /50 Mgm. Dry Wt.	Ratio D/C
A. Tissues					
1) leaf disks	C	24	5.9	9.8	2.0
	D			20.1	
2) stem slices	C	19	6.5	3.3	1.24
	D			4.1	
3) stem slices	C	24	6.5	3.8	1.29
	D			4.9	
				γ Uptake of Indole/Ml. /Mgm. Protein Nitrogen	
B. Extracts					
1) leaf	C	2	7.2	12.4	2.5
	D			30.5	
2) stem	C	2	5.9	68	1.37
	D			93	

The above results clearly show that zinc deficient material is more active in the uptake of indole than the healthy material. Because simultaneous measurement of products was not successful, two interpretations are possible.

1) If indole is converted only to tryptophan, then the tryptophan synthease had enhanced activity under conditions of zinc deficiency. (As has already been noted, increased enzyme activity is a common finding in zinc deficient material). If this is the case, the supposition that the conversion of indole to tryptophan is the precise reaction affected by zinc deficiency is disproved, and it can be concluded that the metabolism of higher plants is different to that of Neurospora in this respect.

2) If enzymes are present in the tomato plant tissues which catalyze the conversion of indole to products other than tryptophan, then increased activity of these enzymes would increase the uptake of indole and yet reduce the formation of tryptophan.

Evidence so far obtained indicates that the first possibility is more likely to be correct. This evidence was obtained by comparing the efficiency of conversion of indole to IAA by zinc deficient and healthy material.

The formation of IAA was calculated as percentage of the indole taken up, the comparison being on a molar basis.

Table 4. Formation of IAA from Indole

	Moles IAA formed as % of theoretical maximum
Experiment 1	
Leaf extract C	78
D	73
Experiment 2	
Leaf extract C	86
D	101

In spite of the reservations already made as to the use of IAA formation as a measurement of tryptophan synthease, the above results suggest that the pathway of indole metabolism is not significantly altered from a qualitative point of view. Any change in the uptake of indole may therefore be expected to result in a corresponding change in the formation of tryptophan and IAA.

If indole is more efficiently converted to tryptophan in zinc deficient plants, how can the reduced content of tryptophan be explained? The most plausible explanation is that the content of indole is reduced. To test this possibility it is necessary to analyse zinc deficient and healthy tomato plant tissues for indole, and to measure the rates of formation of indole. This investigation has not yet been carried out. An attempt to extract "free indole" by grinding leaves with several changes of toluene and testing the toluene extract for indole indicated that any indole present in the tissues is present in bound form, which would only be released on hydrolysis. The precursor of indole in higher plants is not known. To discover it would require an independent investigation, though a convenient starting point would be to test anthranilic acid, which is the precursor of indole in Neurospora (Tatum and Bonner, 59).

III. The Utilization of Tryptophan

a) The Conversion of Tryptophan to Products Other than IAA

Wiltshire (71) has reported the oxidation of tryptophan in pea seedling and tissue extracts. He found the enzyme to be peroxidase, being stimulated by peroxigenic substrates such as putrescine and ethanolamine. Now it is known that the activity of peroxidase is increased in zinc deficient material (Nason et al., 42; Bean, 4), and it was considered that the increased activity may affect the content of tryptophan. If the peroxidase breaking down tryptophan is more active in zinc deficient material, this would

account for the reduced content of tryptophan directly, and indirectly for the reduced amount of IAA.

This possibility was tested.

Experiments were carried out in the Warburg respirometer as outlined in Materials and Methods. The reaction mixture was buffered at pH 6.7 with phosphate buffer in all cases. In various experiments, tryptophan alone was used as the substrate, and in others different compounds were added to stimulate the production of hydrogen peroxide, and so hasten the oxidation of tryptophan if any took place.

The addition of tryptophan alone or in combination with one or more of the compounds mentioned failed to raise the oxygen uptake above the endogenous level. This lack of influence of tryptophan on the uptake of oxygen was the same no matter whether the extract had been prepared from zinc deficient or healthy material. It may therefore be concluded that the peroxidative destruction of tryptophan was not operative in the extracts studied.

Measurements of gas exchange in the above experiments gave no indication of the fate of the added tryptophan. A measure of the utilization of tryptophan and the formation of IAA was obtained by taking aliquots from the vessels at the end of a run and performing colorimetric tests on them. These tests showed that tryptophan was being utilized by the extracts, and that the products included IAA. It was not possible to put the conversion of tryptophan to IAA on a quantitative basis because no colorimetric readings at zero time had been taken. However, the qualitative nature of the conversion can be seen from Table 5. Utilization of tryptophan is indicated by the approach of the value of per cent transmittance to the value of the endogenous solution; formation of IAA is indicated by the deviation of the value for per cent transmittance from

the value for the endogenous solution.

Table 5. Utilization of Tryptophan

(Numerical values are per cent transmittance)

	Tryptophan	IAA
Control		
Endogenous	88.0	82.5
Endogenous + tryptophan	82.5	16.0
Endogenous + tryptophan + ethanolamine	80.5	15.5
Deficient		
Endogenous	84.0	85.5
Endogenous + tryptophan	70.5	70.0
Endogenous + tryptophan + ethanolamine	71.0	70.5

Though the control extract clearly synthesized more IAA than the deficient extract, it is not possible to compare the relative efficiencies because no determinations were made of dry weight or protein nitrogen. Relative efficiencies in the conversion of tryptophan to IAA are discussed in the next section.

b) The Effect of Zinc Deficiency on the Conversion of Tryptophan to IAA Tsui (62) reported that zinc deficiency does not affect the conversion of tryptophan to IAA. His experiments involved incubating leaf disks with tryptophan, and then drying the disks, extracting the auxin with ether and then assaying the auxin by the *Avena* method.

When the production of IAA was being considered as a method of assay for tryptophan synthease, it was essential to know if the zinc deficient and healthy material had equal ability to convert tryptophan to IAA. If they were in fact different, then the production of IAA would be

no measure of tryptophan synthease. This point was of such importance that the finding of Tsui was checked, using colorimetric methods instead of the biological assay.

The experimental material was incubated with phosphate-citrate buffer, and tryptophan, in 20 ml. volume. At the beginning of the experiment, and at the end, aliquots were taken for colorimetric analysis for IAA. The results of two experiments are gathered in the following table.

Table 6. Formation of IAA from Tryptophan

Material		Time Incubated (hours)	pH	γ IAA Formed/Ml./ 50 Mgm. Dry Wt.	D/C x 100
Leaf disks	C	17	6.5	4.6	96
	D			4.4	
				γ IAA Formed/Ml./ Mgm. Protein Nitrogen	
Leaf extract	C	12	7.2	124	89
	D			110	

From the above results it is concluded that the formation of IAA from tryptophan is not significantly changed under conditions of zinc deficiency, supporting the earlier discovery of Tsui.

IV. Indoleacetic Acid Oxidase

Several pieces of evidence have been brought forward in the Literature Review to suggest that the reduction in the content of IAA in zinc deficient plants is due to increased activity of IAA oxidase. Preliminary experiments by Skoog (50) indicate that this is the case.

In the work described here the enzyme was first looked for in tomato plants, and some of its characteristics investigated to see how well it compared with the enzyme described from other sources.

Presence of the Enzyme in Tomato Plant Tissues A naturally occurring inhibitor of IAA oxidase is known, and it is particularly concentrated in the green parts of the plant (Galston and Baker, 18). In the tomato plant IAA oxidase was never found in the green parts of the plant, but activity was frequently found in root homogenates. The presence of the enzyme was demonstrated by the colorimetric technique of Tang and Bonner (57), and by the manometric technique of Wagenknecht and Burris (67). However, the enzyme was not found consistently; in some cases the addition of IAA to extracts inhibited rather than stimulated respiration. No explanation for this erratic behavior was found.

Effect of Substrate Concentration The effect of three concentrations of IAA on the oxygen uptake in the Warburg respirometer is shown in Figure 10. The vessels contained 1.0 ml. root extract; phosphate buffer pH 6.3; and IAA solution. Increasing concentrations of IAA clearly stimulate increased oxygen uptake.

Effect of Inorganic Ions Previous reports of the effect of manganous ions on IAA oxidase have been conflicting. The enzyme from etiolated pea seedlings is inhibited by manganese ions (Galston and Baker, 18), while the enzyme from the roots of the wax bean is stimulated by these ions (Wagenknecht and Burris (67). The effect on the enzyme from tomato roots was tested, and found to be stimulated by manganous chloride at concentrations in the region of 10^{-4} M. At the same time the effect of zinc and copper ions was also tested. The solutions were added to the Warburg vessels so that their final concentration was 10^{-4} M. The results are shown in Table 7.

FIGURE 10. EFFECT OF IAA ON TOMATO ROOT RESPIRATION

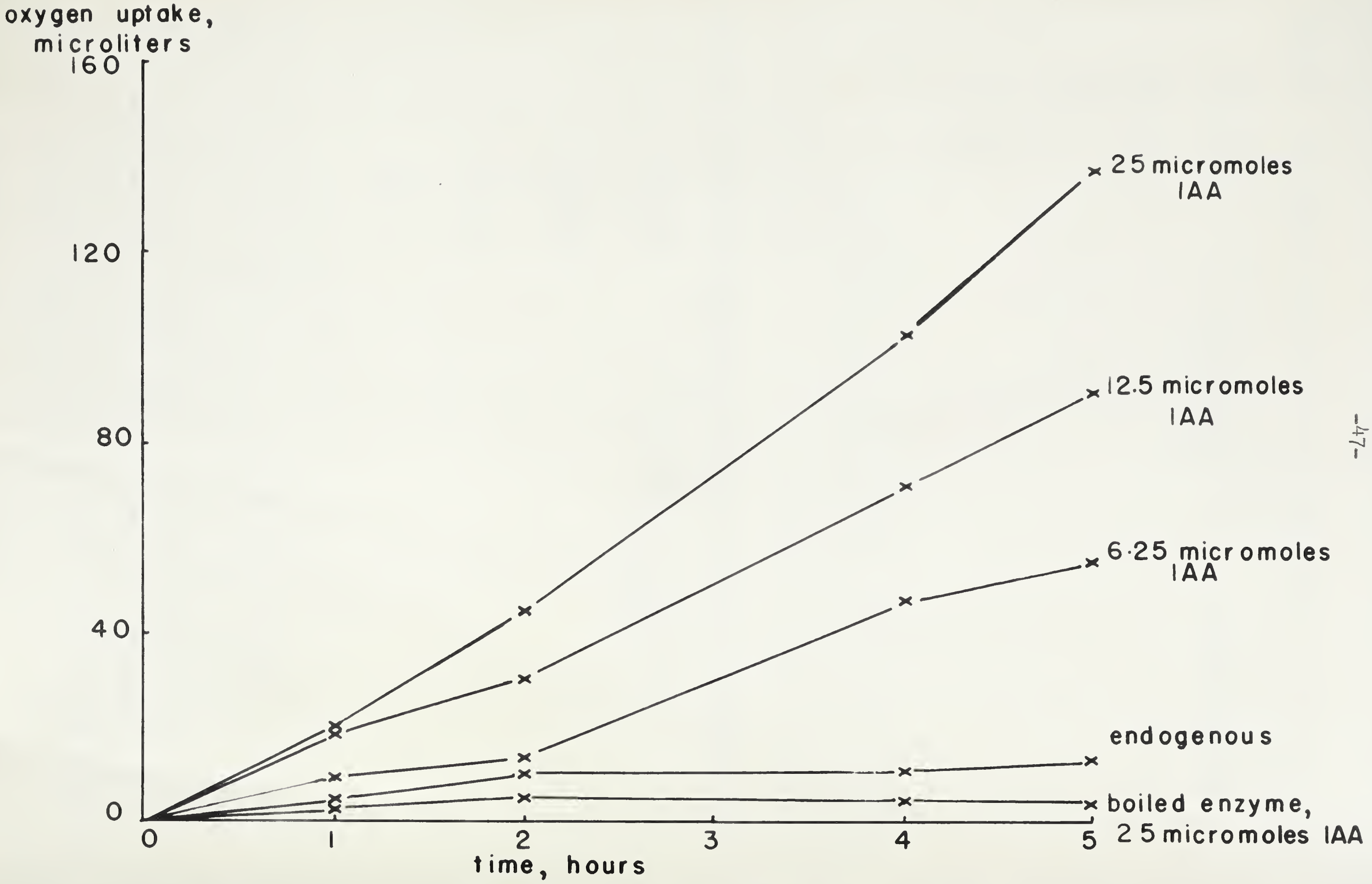


Table 7. The Effect of Inorganic Ions on the Activity of IAA Oxidase (a)

Treatment	Oxygen Uptake After $5\frac{1}{4}$ hrs. (Microliters)	% Change from IAA
Endogenous	7.0	---
IAA	51.0	---
IAA, Mn	63.0	24
IAA, Cu	9.0	-84
IAA, Zn	50.0	---

The inorganic ions had no effect on endogenous respiration.

Some preparations showed very little ability to oxidize IAA. The effect of inorganic ions on such a preparation is shown in Table 8.

Table 8. The Effect of Inorganic Ions on the Activity of IAA Oxidase (b)

Treatment	Oxygen Uptake After $4\frac{1}{2}$ hrs. (Microliters)
Endogenous	10
IAA	8
IAA, Mn	30
IAA, Cu	10
IAA, Zn	10

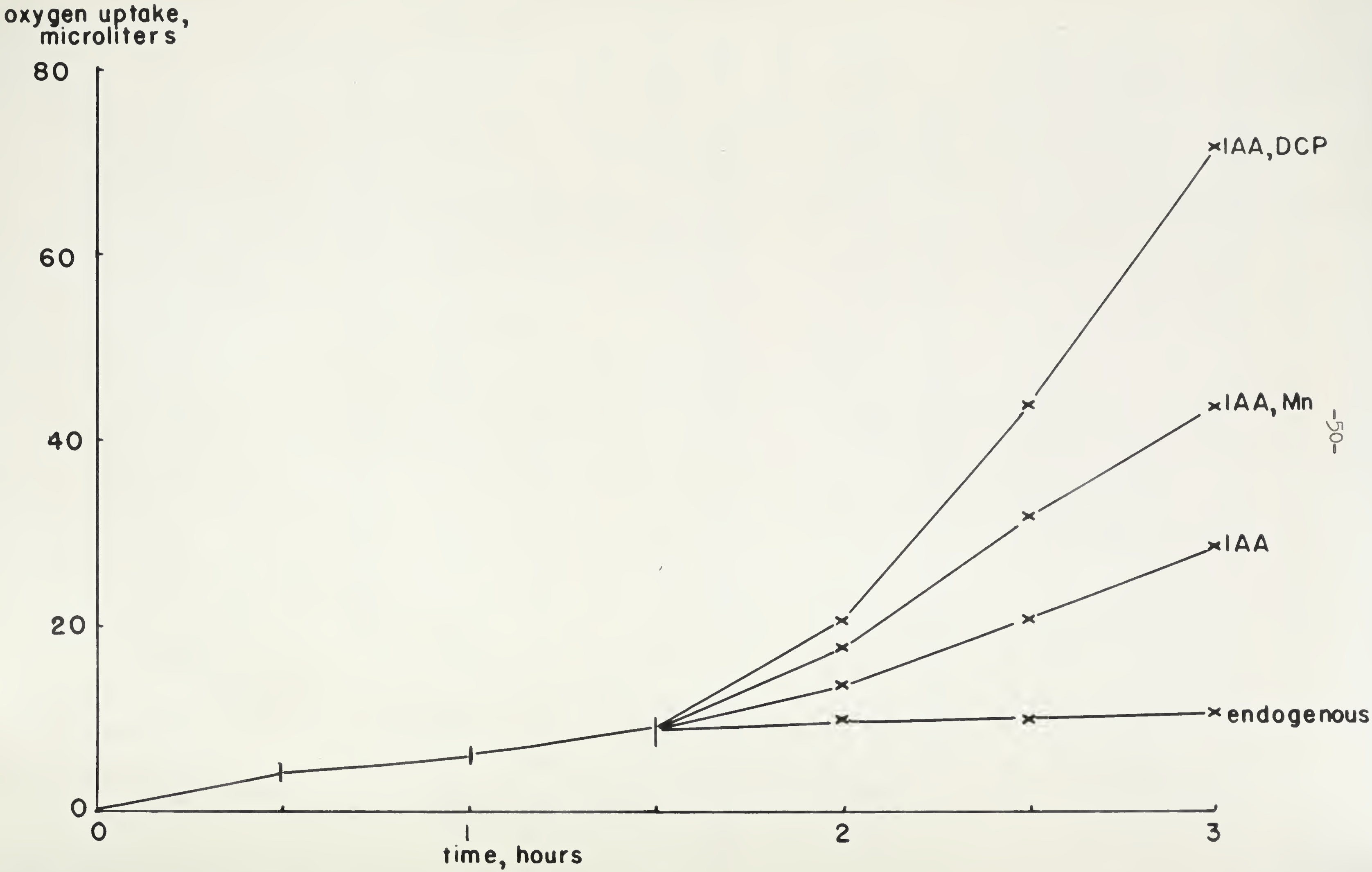
When considered together the two experiments indicate that the IAA oxidase from tomato roots is stimulated by manganous ions, inhibited by copper ions, and is unaffected by zinc ions. The inhibition by copper is evidence that the activity of the enzyme system is dependent upon sulfhydryl groups; James (32) has stated that copper ions at low concentrations have been found to inhibit enzymes containing the sulfhydryl group as a necessary factor for activity.

The fact that oxygen uptake measured destruction of IAA was checked by colorimetric analysis of aliquots of the reaction mixture taken from the Warburg vessels at the end of a run. Oxygen uptake varied positively with the destruction of IAA as measured by colorimetric analysis.

Effect of DCP (2,4-Dichlorophenol) IAA oxidase has been found to be stimulated by DCP, (Goldacre, Galston, and Weintraub, 25). Experiments with this compound showed that a similar effect is produced with the enzyme from tomato roots. Results are presented in Figure 11. In this experiment a comparison was made of the relative effectiveness of manganese ions and DCP, both at final concentrations in the Warburg vessels of 10^{-4} M. An interesting point in the graphs of Figure 11 is that stimulation of oxygen uptake did not occur until a period of 1½ hours had elapsed. Vertical lines at ½, 1 and 1½ hours indicate the range between the highest and lowest values. This period can be conjectured as a limitation of the supply of hydrogen peroxide. Where IAA alone is the substrate, the period indicates that hydrogen peroxide is formed from IAA as has been suggested by Galston, Bonner and Baker (21), and confirmed by Siegel and Galston (49). The fact that DCP did not remove the lag period suggests that its stimulatory effect is on the peroxidase step of the IAA oxidase enzyme system. This is in agreement with the finding of Lockhart (38).

Relationship of Polyphenol Oxidase and IAA Oxidase Thimann (60) has presented evidence suggesting that inactivation of growth hormone by leaf extracts is due to oxidation by a peroxidase-catechol oxidase system. Experiments were conducted with the object of testing the relationship of polyphenol oxidase activity to IAA oxidase activity. These experiments were carried out in the Warburg respirometer. IAA oxidase activity and

FIGURE 11. STIMULATION OF IAA OXIDASE



polyphenol oxidase activity were measured simultaneously with control and zinc deficient extracts. Results of the experiments are shown in Table 9.

Though the zinc deficient material showed greater activity in the oxidation of catechol, no indication of enhanced destruction of IAA was detected. However, neither control nor zinc deficient extracts showed ability to oxidize IAA. For this reason no final judgement on the relationship of polyphenol oxidase and IAA oxidase can be made.

Table 9. Relationship of Polyphenol Oxidase and IAA Oxidase

Final concentrations in Warburg vessels: catechol, 10^{-3}M, (added from side arm at zero time); IAA, 1.6 10^{-3}M. Figures are oxygen uptake in microliters).

Time	C end	D end	C + Catechol	D + Catechol	C + IAA	D + IAA
20 min.	5.0	4.0	25.0	40.0	--	--
2 hrs.	18	13	----	----	12	10

The greater activity of the deficient extract in the oxidation of catechol is a direct indication of superior specific activity as both enzyme extracts contained 8 mgm. dry matter per ml. This finding is in agreement with the report of Nason et al. (42), that polyphenol oxidase activity is increased in zinc deficient plants.

DISCUSSION

The discovery that zinc deficiency specifically reduces the activity of the enzyme responsible for the synthesis of tryptophan in Neurospora, has directed interest to the possibility of a similar effect of zinc deficiency in higher plants (McElroy and Nason, 39). The suggestion was made in spite of the fact that, at the time, no enzyme catalyzing the synthesis of tryptophan in higher plants had been reported. However, the knowledge that the content of tryptophan is reduced in zinc deficient plants was strong circumstantial evidence in support of the hypothesis (Tsui, 62).

Experiments reported in this thesis show that indole is definitely a metabolite of higher plants. The dependency of indole uptake on the presence of serine which has been demonstrated with tomato cotyledons indicates that the utilization of indole in higher plants is similar to the utilization in Neurospora. That is to say, indole and serine are combined by a chemical condensation. Further evidence that the reaction is the same has been obtained by the detection of tryptophan as a product of the reaction. By changing the pH of the reaction mixture, IAA has also been demonstrated as a reaction product. One unsatisfactory feature of the work on tryptophan synthease has been the inability to measure the quantities of tryptophan produced when indole was taken up. This was because of the limited sensitivity of the color test for tryptophan.

The finding that zinc deficient material is more efficient in taking up indole is in direct contrast to the finding with Neurospora, and appears to disprove the hypothesis of McElroy and Nason (39). However, some reservations must be made. Because it was not possible to determine the relative efficiency of the zinc deficient and healthy material in the

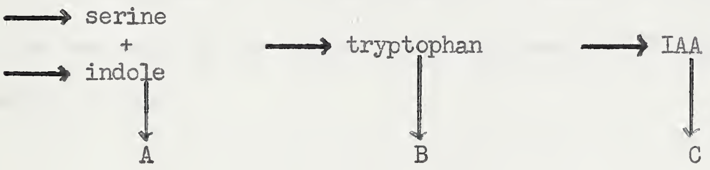
synthesis of tryptophan, it could not be concluded that zinc deficiency does not affect the pathway of indole metabolism. Evidence is given in the experimental section that strongly suggests that the pathway is not altered, but for complete satisfaction it would be preferable to manipulate the experimental conditions so that tryptophan could be determined quantitatively. The tryptophan test as used would be satisfactory if there were less turbidity in the reaction mixture, and the turbidity could be reduced by using concentrated and purified enzyme preparations. But for purposes of comparison of zinc deficient and healthy material, such preparations would not be as valuable as crude preparations since differences found might be attributable to the methods of purification rather than to true physiological differences.

If, on the basis of the available evidence, it is accepted that zinc deficient material is more efficient than healthy material in the conversion of indole to tryptophan, what conclusions may be drawn? If indole were equally available in the two cases then the zinc deficient material should contain more tryptophan. This is known not to be the case. This line of reasoning suggests that the zinc deficient material should contain less indole - a theory which has yet to be tested. If it is correct, the effect of zinc deficiency on the synthesis of IAA must be on some reaction leading to the formation of indole. It is tentatively suggested that indole is formed from anthranilic acid. This idea can also be readily tested, and will give further information about the influence of zinc deficiency on the metabolism of IAA in higher plants.

Wiltshire's (71) report that an enzyme derived from etiolated pea epicotyls catalyzes the peroxidative destruction of tryptophan raised the possibility that the conversion of tryptophan to products other than IAA may take place under conditions of zinc deficiency. Such a

diversion would account for the observed effect of zinc deficiency on the content of tryptophan and IAA. Experiments reported in this thesis showed that peroxidative destruction of tryptophan did not take place under the conditions employed.

The idea implicit in the preceding discussion may be emphasized by the following diagram, which shows the metabolic pathway leading to the formation of IAA, and hypothetical side reactions which would reduce the supply of IAA precursors.



In the diagram, the known reactions in normal plants are indicated as solid horizontal arrows. Any break in the reactions symbolized by these arrows would account for the observation that IAA concentration is lowered in zinc deficient plants. Tsui (62) has reported that the conversion of tryptophan to IAA is unaffected in zinc deficient plants and this has been endorsed by experiments reported in this thesis. The changes discovered in the activity of the enzyme responsible for the conversion of indole to tryptophan also do not account for the reduced content of IAA. Effects of zinc deficiency on the synthesis of indole remain to be tested.

The side reactions A, B, and C should be considered in the discussion of zinc deficiency. Increase in the activity of the enzymes A, B and C, would account for the observed effect of zinc deficiency on IAA content. A is an entirely hypothetical enzyme. B corresponds to the enzyme described by Wiltshire (71); as mentioned above it does not seem to be operative in tissue extracts of tomato leaves and so may be dismissed as being the point of influence of zinc deficiency. The third enzyme C

corresponds to IAA oxidase. Preliminary experiments by Skoog (50) suggested that this enzyme is more active under conditions of zinc deficiency. Attempts to extend the finding of Skoog have so far been unsuccessful, but the reaction still deserves attention as a possible solution of the problem of zinc deficiency.

The compounds taking part in the metabolic pathway leading to the formation of IAA are now known to be numerous and to stretch from indole to IAA. In the study of the effects of zinc deficiency of these reactions it now appears that the likeliest avenues of further research are at the two ends of the chain; - the synthesis of indole, and the destruction of IAA.

SUMMARY

An enzyme catalyzing the uptake of indole has been found in tomato plant tissues and tissue extracts. It has been concluded that the uptake of indole is by the same mechanism as that found in *Neurospora*. This conclusion was reached after tryptophan and IAA were identified as products of the reaction, and when, in some cases, serine was required for indole uptake. Several of the characterizing properties of the enzyme have been worked out; among these the pH optimum is quite low - about pH 5.0.

Comparison of zinc deficient and healthy plant material, on a dry weight or protein nitrogen basis, showed the zinc deficient material to be more efficient in the uptake of indole. This indicates that zinc deficiency affects higher plants and *Neurospora* in a different manner.

No indication of the peroxidative destruction of tryptophan was found in zinc deficient or healthy material. It therefore appears that the reduced content of tryptophan and IAA can not be accounted for by changes in the activity of this enzyme.

The absence of effect of zinc deficiency on the conversion of tryptophan to IAA has been reconfirmed.

Attempts to obtain a comparison of the relative efficiencies of IAA oxidase in zinc deficient and healthy plants were unsuccessful.

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